

Reduction in Nerve Growth Factor Availability Leads to a Conditioning Lesion-like Effect in Sympathetic Neurons

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ABSTRACT: Axotomized peripheral neurons are capable of regeneration, and the rate of regeneration can be enhanced by a conditioning lesion (i.e., a lesion prior to the lesion after which neurite outgrowth is measured). A possible signal that could trigger the conditioning lesion effect is the reduction in availability of a target-derived factor resulting from the disconnection of a neuron from its target tissue. We tested this hypothesis with respect to nerve growth factor (NGF) and sympathetic neurons by administering an antiserum to NGF to adult mice for 7 days prior to explantation or dissociation of the superior cervical ganglion (SCG) and subsequently measuring neurite outgrowth. The antiserum treatment dramatically lowered the concentration of NGF in the SCG and increased the rate of neurite outgrowth in both explants and cell cultures. The increase in neurite outgrowth was similar in magnitude to that seen after a conditioning lesion. To determine if ex-

ogenous NGF could block the effect of a conditioning lesion, mice were injected with NGF or cytochrome C immediately prior to unilateral axotomy of the SCG, and for 7 days thereafter. A conditioning lesion effect of similar magnitude was seen in NGF-treated and control animals. While NGF treatment increased NGF levels in the contralateral control ganglion, it did not significantly elevate levels in the axotomized ganglion. The results suggest that the decreased availability of NGF after axotomy is a sufficient stimulus to induce the conditioning lesion effect in sympathetic neurons. While NGF administration did not prevent the conditioning lesion effect, this may be due to the markedly decreased ability of sympathetic neurons to accumulate the growth factor after axotomy. © 2006 Wiley Periodicals, Inc. *J Neurobiol* 66: 000–000, 2006

Keywords: axotomy; nerve growth factor; nerve injury; regeneration; superior cervical ganglion

INTRODUCTION

Peripheral neurons are capable of axonal regeneration following axotomy, whereas injured neurons of the central nervous system (CNS) show little or no regeneration (Ramon y Cajal, 1928; Guth, 1956). It is of in-

terest to understand the mechanisms that enable peripheral neurons to regenerate in order to identify ways to improve this regeneration and to promote regeneration in the CNS. An effective way to enhance the rate of nerve regeneration in sensory and motor neurons is to induce a lesion (i.e., a conditioning lesion) prior in time to the lesion after which neurite outgrowth is measured (i.e., a test lesion; McQuarrie and Grafstein, 1973). A conditioning lesion not only enhances peripheral nerve regeneration, but also stimulates regeneration of the central branch of sensory neurons into and within the CNS, an environment normally inhibitory to regeneration (Richardson and Verge, 1987; Neumann and Woolf, 1999).

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We recently demonstrated that like sensory and motor neurons, sympathetic neurons exhibit an increase in their rate of regeneration after a conditioning lesion (Shoemaker et al., 2005a). This effect was seen both following a lesion proximal to the neurons' cell bodies and following a distal lesion produced by target organ removal. In the present study, we asked what the signal might be that triggered this regenerative effect.

The conditioning lesion effect is one of a number of neuronal changes that occurs as a result of axotomy. In general two types of signals have been postulated to underlie these changes: positive signals that are induced by the injury and promote a regenerative response, and the elimination of negative signals originating from the peripheral target that normally exert a constitutive inhibitory influence on regeneration. The cytokine leukemia inhibitory factor (LIF) is an example of one such positive factor. Though not detectable in the intact nervous system, LIF mRNA is induced following axotomy in non-neuronal cells at the site of injury (Curtis et al., 1994; Banner and Patterson, 1994; Sun and Zigmond, 1996b) and within axotomized sympathetic ganglia (Sun et al., 1994, 1996a). An aspect of the neuronal response to axotomy, the up-regulation of gene expression of certain neuropeptides (e.g., galanin), was attenuated in axotomized sympathetic and sensory ganglia of LIF null mice compared to those of wild-type controls (Rao et al., 1993; Sun and Zigmond, 1996a,b). In addition, exogenous LIF enhances regeneration of axotomized sensory and motor axons *in vivo* (Tham et al., 1997; Hart et al., 2003; Zang and Cheema, 2003). Most relevant to our study, LIF null mice exhibit an attenuation of the conditioning lesion effect observed in sensory neurons (Cafferty et al., 2001). Nevertheless, we found that, in sympathetic neurons, LIF is not necessary for the conditioning lesion effect (Shoemaker et al., 2005a).

The present study focuses on a possible negative signal, namely, a target-derived trophic factor. According to the classical view of neurotrophins, they are expressed by peripheral target tissues, are released by these tissues, and bind to neurotrophin receptors on peripheral nerve terminals (for review see Thoenen and Barde, 1980). Nerve growth factor (NGF) and its high affinity receptor *trkA* are endocytosed by sympathetic nerve terminals and then retrogradely transported to the cell body where the signaling pathways activated produce modifications in gene transcription (Palmatier et al., 1984; Segal and Greenberg, 1996; Riccio et al., 1997, 1999). Axotomy leads to disconnection of innervating neurons from their target tissues and eliminates access of axons to target-derived

neurotrophins (Korsching and Thoenen, 1985; Nagata et al., 1987; Zhou et al., 1994).

The reduction of NGF availability after axotomy has been implicated in several of the axotomy-induced changes in gene expression in adult peripheral neurons. In sympathetic neurons, administration of antiserum to NGF (anti-NGF) to unoperated animals increases expression of galanin, vasoactive intestinal peptide, and activating transcription factor 3 (ATF3; Shadiack et al., 1998, 2001; Hyatt Sachs et al., 2005) and decreases expression of neuropeptide Y, tyrosine hydroxylase, and the low affinity neurotrophin receptor p75 (Zhou and Rush, 1996; Shadiack et al., 2001; Hyatt Sachs et al., 2005). In sensory neurons, the changes produced by anti-NGF include increased expression of galanin, vasoactive intestinal peptide, c-jun, and damage-induced neuronal endopeptidase (Gold et al., 1993; Shadiack et al., 2001; Kato et al., 2002; Csillik et al., 2003) and decreased expression of substance P and calcitonin gene-related peptide (Shadiack et al., 2001). In addition, the expression of certain axotomy-induced genes (e.g., galanin and ATF3) in sympathetic and sensory neurons is attenuated by the administration of exogenous NGF (Verge et al., 1995; Shadiack et al., 2001; Averill et al., 2004). Together, these experiments suggest that NGF functions as a negative factor that is capable of inhibiting axotomy-induced changes in gene expression.

We hypothesize that reduction of NGF in neurons not only induces changes in the expression of genes associated with axotomy, but ultimately results in events that stimulate peripheral nerve regeneration. In support of this hypothesis, previous studies using colchicine or vinblastine treatment to block axonal transport suggest that the reduction in an unidentified target-derived factor induces an increase in sensory neurite outgrowth subsequently measured *in vitro* (White et al., 1996; Smith and Skene, 1997). The present study examines whether the reduction in NGF availability induces a regenerative response in sympathetic neurons of the mouse superior cervical ganglion (SCG).

To reduce levels of NGF available to sympathetic neurons, daily injections of anti-NGF were administered. The regenerative potential of sympathetic neurons was assessed by measuring neurite outgrowth in SCG explants and dissociated cell cultures. We have recently characterized the increased rate of outgrowth by sympathetic neurons following axotomy 1 week prior to explantation or dissociation of the SCG (Shoemaker et al., 2005a). For comparison purposes, in the present experiments the levels of NGF in the ganglion were reduced by administration of NGF antiserum for 1 week prior to explantation or dissoci-

ation. In addition, we determined whether the administration of NGF following axotomy *in vivo* would eliminate or reduce the conditioning lesion effect induced by axotomy. Portions of these data have been presented in abstract form (Shoemaker et al., 2005b).

MATERIALS AND METHODS

Animal Injections

Adult (8 weeks) male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were used for all experiments. Over a 7 day period, mice received daily intraperitoneal (IP) injections of anti-NGF (obtained from J. Diamond; see Diamond et al., 1987; Gloster and Diamond, 1992) given at one of two doses referred to as the lower dose (2.5 $\mu\text{L/g}$) or the higher dose (7.5 $\mu\text{L/g}$). Control animals were injected with normal sheep serum (NSS; Sigma-Aldrich Co., St. Louis, MO) given at the higher dose only (7.5 $\mu\text{L/g}$). Anti-NGF and NSS were diluted with 0.9% saline for a total injection volume of 10 $\mu\text{L/g}$ body weight. After 7 days of injections, ganglia were removed and explanted (low anti-NGF, $n = 5$; high anti-NGF, $n = 10$; NSS, $n = 10$), dissociated (high anti-NGF, $n = 7$; NSS, $n = 6$), or frozen for detection of NGF protein levels using enzyme-linked immunosorbant assay (ELISA; low anti-NGF, $n = 6$; high anti-NGF, $n = 6$; NSS, $n = 6$). An additional group of mice that were not injected but had their SCG axotomized unilaterally were included in the explant and ELISA experiments (see below).

In a separate set of experiments, mice received daily IP injections of NGF (Mouse Natural β -NGF; Austral Biologics, San Ramon, CA) or cytochrome C (Sigma-Aldrich Co.) given at 1 $\mu\text{g/g}$ body weight for 7 days. Both NGF and cytochrome C were reconstituted in 0.1 M phosphate buffered saline (PBS) at a concentration of 0.1 $\mu\text{g}/\mu\text{L}$ and a total injection volume of 10 $\mu\text{L/g}$ body weight was administered. Immediately after the first injection of NGF or cytochrome C, mice were anesthetized and underwent a unilateral axotomy of the postganglionic nerves of the SCG (see below). Mice received six additional daily injections of NGF or cytochrome C. The day following the last injection, SCG were dissected and explanted (NGF, $n = 8$; cytochrome C, $n = 10$) or frozen for detection of NGF protein by ELISA (NGF, $n = 5$; cytochrome C, $n = 5$).

Animal Surgeries

Mice were anesthetized with an IP injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). Unilateral axotomy was performed by transecting the postganglionic external and internal carotid nerves. The postganglionic nerves of the SCG on the contralateral side were exposed but not injured providing a sham-operated control ganglion. One week following surgery, ganglia were dissected and explanted (axotomy, $n = 5$; sham-operated, $n = 5$) or frozen for detection of NGF levels by ELISA (axotomy, $n = 5$; sham-operated, $n = 6$).

SCG Explantation

One week following nerve injury or injections, mice were sacrificed by CO_2 inhalation. SCG were removed and explanted in 7.5 μL Matrigel (solubilized basement membrane from EHS mouse sarcoma cells; Becton Dickinson, Franklin Lakes, NJ) using F12 medium (Invitrogen, Carlsbad, CA) containing 5 mg/mL bovine serum albumin, 5 $\mu\text{g}/\text{mL}$ insulin, 10 U/mL penicillin, 10 $\mu\text{g}/\text{mL}$ streptomycin, 0.63 $\mu\text{g}/\text{mL}$ progesterone, 8.8 $\mu\text{g}/\text{mL}$ putrescine, 0.005 $\mu\text{g}/\text{mL}$ selenium, and 100 $\mu\text{g}/\text{mL}$ transferrin as described in Shoemaker et al. (2005a). NGF was not added to the medium. Phase-contrast images of neurite outgrowth from each SCG were captured at 6, 12, 18, 24, and 48 h after explantation using an Axiovert 405M microscope (10X magnification). Following imaging at 48 h, SCG were fixed with 4% paraformaldehyde for 1 h. In order to obtain optimal visualization of the neuronal processes in the fixed explants, immunohistochemistry for β III tubulin-immunoreactivity was performed in fixed whole-mount SCG following the procedure of Shoemaker et al. (2005a).

Explant Analysis

Neurite outgrowth was assessed using Metamorph software (version 4.6; Universal Imaging Corporation, Downingtown, PA) as described previously (Shoemaker et al., 2005a). At each time point described above, the distances between the edge of the ganglion and the leading tip of the furthest five processes observed in phase microscopic images were measured in each explant. A minimum of four ganglia was included for each experimental group. The mean \pm standard error of the mean of the five longest neurites is shown for each treatment group. In two experiments, results were obtained also from measuring the 30 longest processes and the data were compared to those obtained from measuring the five longest processes. The shapes of the growth curves for each experimental group were nearly identical using the two methods, and the statistical significance of differences between groups was the same.

The rate of elongation and the delay in the initiation of neurite outgrowth (initial delay) were determined from a regression analysis in which the data were fit to a straight line. Regression coefficients revealed a linear correlation ($r^2 > 0.95$) between neurite length and time in culture. The slope of the linear function was taken as the rate of elongation, and the intercept on the x axis as the initial delay in outgrowth (Shoemaker et al., 2005a).

Dissociated SCG Neurons

Dissociated SCG neurons were obtained from mice after 7 days of injection with 7.5 $\mu\text{L/g}$ NSS ($n = 6$) or anti-NGF serum ($n = 7$). Mice were sacrificed by CO_2 inhalation, and SCG neurons were dissociated and cultured using a modification of the procedure described by Orike et al. (2001a) and Shoemaker et al. (2005a). Briefly, the SCG were removed and placed in cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's Balanced Salt Solution (HBSS; GIBCO/Invitrogen) buffered

with 10 mM HEPES (Sigma-Aldrich Co.). The SCG were desheathed, trimmed of all visible trunks, and each SCG was cut into six small pieces. The SCG pieces were digested at 37°C with 1 mg/mL collagenase (Sigma-Aldrich Co.) for 40 min and washed in HBSS/HEPES followed by digestion in 1 mg/mL trypsin (Sigma-Aldrich Co.) for 10 min followed by washing in HBSS/HEPES. Dissociation and culture of the SCG neurons was carried out in defined serum-free Ham's F-14 medium (JRH Biologicals, Lenexa, KS) supplemented with 0.35% Path-4-BSA (MP Biomedicals, Inc., Aurora, OH), 60 ng/mL progesterone, 16 $\mu\text{g}/\text{mL}$ putrescine, 400 ng/mL L-thyroxine, 38 ng/mL sodium selenite, 340 ng/mL tri-iodothyronine, 2 mM L-glutamine, 60 $\mu\text{g}/\text{mL}$ penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (all from Sigma-Aldrich Co.). No growth factors or mitotic inhibitors were included in the medium. The SCG pieces were dissociated by trituration with a Pasteur pipette treated with Sigmacote (Sigma-Aldrich Co.), pulled to an internal bore diameter of 0.05 to 0.1 mm, and fire polished. The dissociated neurons were gently dispersed onto 22 mm glass coverslips coated with 0.01% poly-L-ornithine (Sigma-Aldrich Co.) and 20 $\mu\text{g}/\text{mL}$ laminin (Roche Diagnostics Corp., Indianapolis, IN) in six-well tissue culture plates containing 2 mL F-14 medium such that each well contained the equivalent of one-half of an SCG. This resulted in a low density of cells that minimized any overlap of individual cells and processes. Cells were cultured for 24 h at 37°C in 95% air/5% CO₂.

Immunohistochemistry and Analysis of Dissociated SCG Neurons

Twenty-four hours after plating dissociated SCG neurons, coverslips containing the cells were fixed in 4% paraformaldehyde in PBS for 20 min followed by three washes in PBS. In order to visualize neurons and processes, the cultures were labeled with anti- β III tubulin monoclonal antibody (1:1000; Promega, Madison, WI) followed by CY3-conjugated donkey antimouse IgG, F(ab')₂ fragment (1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The glass coverslips were inverted and mounted on glass slides with Gel/Mount mounting media (Biomedex, Foster City, CA).

Cells that were β III tubulin-immunoreactive (β III tubulin-IR) were visualized on a Leitz epifluorescence microscope (Leica Microsystems, Inc., Bannockburn, IL), and images were captured with a Hamamatsu ORCA 100 cooled CCD camera (W. Nusbaum, Inc., McHenry, IL) interfaced with C-imaging software (Compix, Inc., Cranberry, PA). Neurite length was measured from the captured images using Metamorph software. The longest neurite was measured from all β III tubulin-IR cells on the glass coverslips that had a process of at least 1.5 times the diameter of the cell body and could be clearly distinguished from any adjacent cell bodies or processes. All data are given as mean values \pm the standard error of the mean.

To analyze the difference between cultured dissociated SCG neurons from mice injected with anti-NGF or NSS, the mean length of the longest neurite per neuron was

determined for all the cells with processes visualized on the coverslips for each SCG. For comparison with the explant data, we also determined the five longest neurites from each group of measured cells per SCG and determined the mean length of those five processes. In addition, the distribution of the lengths of the longest neurite per neuron was analyzed by sorting the neurons into four ranges of neurite length: 0–99, 100–199, 200–299, and 300 μm or higher and determining the percent of the total cells with processes for each SCG that were in each range. The percentage of β III tubulin-IR cell bodies that contained a neurite at least 1.5 times the diameter of the cell body was determined for each SCG for both treatment groups.

Extraction and Quantification of NGF Protein

Individual ganglia were homogenized in 40 μL lysis buffer containing 137 mM NaCl, 20 mM Tris HCl (pH 8.0), 1% Igepal, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, 10 $\mu\text{g}/\text{mL}$ aprotinin, 1 $\mu\text{g}/\text{mL}$ leupeptin, and 0.5 mM sodium vanadate. Total protein concentrations in lysates were determined using a Micro Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Rockford, IL). Each ganglion yielded approximately 25 μg of total protein.

NGF protein levels were assessed in each sample by a two site ELISA using an NGF Emax ImmunoAssay System according to the manufacturer's instructions (Promega). Sample lysates (0.1–1.5 μg protein) and NGF standards (ranging from 7.8 to 250 pg/mL) were loaded in duplicate and, following the immunoassay procedure, absorbances at 450 nm were measured for each well on a V_{Max} microplate reader (Molecular Devices, Sunnyvale, CA). Sample NGF protein concentrations were calculated from the standard curve using SOFTmax PRO software (version 3.0; Molecular Devices). The sensitivity of the assay was defined by an absorbance value two times the blank, giving a sensitivity of 15.6 pg/mL. NGF protein in SCG lysates is presented as pg NGF/ μg protein.

Statistical Analysis

Comparisons between groups were analyzed by performing a Student's *t* test or, where indicated, a one-way analysis of variance (ANOVA) followed by a multiple comparisons test using the Student-Newman-Keuls method.

RESULTS

Following the administration of the lower dose of anti-NGF, neurite outgrowth from the SCG was enhanced after 48 h in explant culture [Fig. 1(C)] compared with that seen in ganglia isolated from mice injected with NSS [Fig. 1(A)]. A similar enhancement of neurite outgrowth was observed following injections of the higher dose of anti-NGF [Fig. 1(D)].

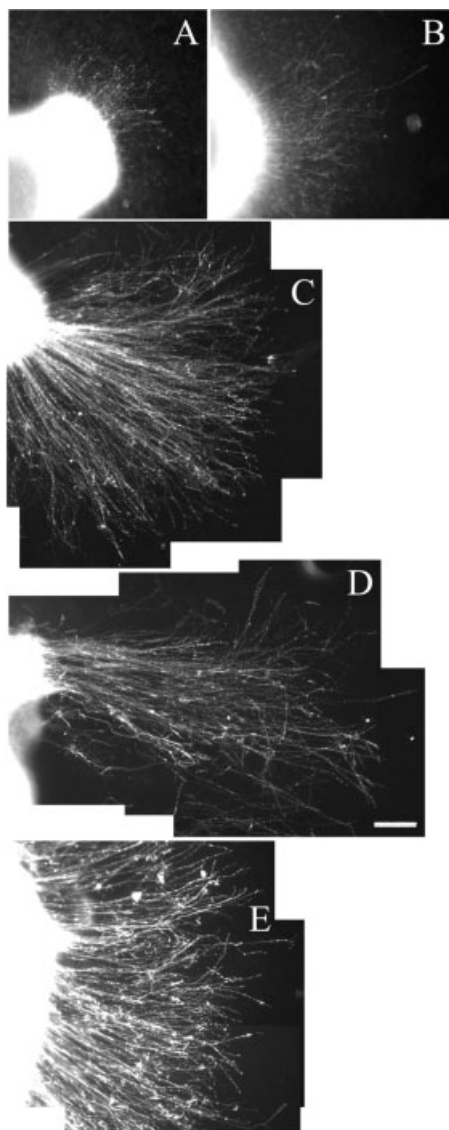


Figure 1 Effect of antiserum to NGF (anti-NGF) or axotomy on sympathetic neurite outgrowth. Adult mice received daily injections of a low dose of anti-NGF ($2.5 \mu\text{L/g}$), a high dose of anti-NGF ($7.5 \mu\text{L/g}$), or normal sheep serum (NSS; $7.5 \mu\text{L/g}$) for 1 week prior to explantation of SCG in Matrigel. Montaged photomicrographs of βIII tubulin-immunoreactive fibers in SCG explants show that sympathetic neurite outgrowth was enhanced 48 h after explantation of ganglia from mice injected with low (C) or high (D) anti-NGF compared to that of ganglia isolated from mice injected with NSS (A). Ganglia axotomized 1 week prior to explantation (E) show increased neurite outgrowth at 48 h after explantation compared to sham-operated ganglia (B). Scale bar = $100 \mu\text{m}$.

This increase in neurite outgrowth was comparable to that observed when SCG axotomized 1 week prior to explantation [Fig. 1(E)] were compared to ganglia from sham-operated mice [Fig. 1(B)]. Neurite length

was quantified at 6, 12, 18, 24, and 48 h following explantation. Ganglia injected with either the lower dose or higher dose of anti-NGF showed a significant increase in neurite length compared to ganglia isolated from mice injected with NSS at all time points examined except 6 h (Fig. 2). As shown previously (Shoemaker et al., 2005a), ganglia explanted 1 week after axotomy also showed a significant increase in neurite length compared to that of sham-operated ganglia at all time points measured (Fig. 2). Estimation of the rate of neurite outgrowth from a regression analysis showed a significant two-fold increase in the rate of outgrowth from ganglia isolated from mice receiving injections of the lower dose ($400 \pm 47 \mu\text{m/day}$) and the higher dose of anti-NGF ($436 \pm 61 \mu\text{m/day}$) compared with that of ganglia from NSS-injected animals [$211 \pm 20 \mu\text{m/day}$; Fig. 3(A)]. A similar increase in rate of outgrowth was observed in

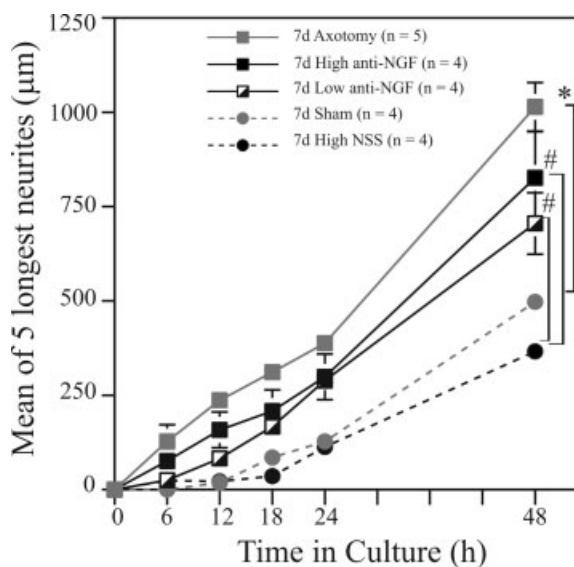


Figure 2 Effect of anti-NGF or axotomy on sympathetic neurite outgrowth over time. Adult mice received seven daily injections of anti-NGF (low dose, $2.5 \mu\text{L/g}$; high dose, $7.5 \mu\text{L/g}$), or had their SCG axotomized unilaterally 1 week prior to explantation of the SCG in Matrigel. Sympathetic neurite length increased in ganglia explanted from mice injected with low or high anti-NGF compared to ganglia isolated from mice injected with NSS. n = the number of explants included in each experimental group. Brackets indicate comparisons where significant differences ($p < 0.05$) were identified between treatments. The asterisks symbol indicates comparisons where significant differences ($p < 0.05$) were found at all five time points examined. The number symbol represents comparisons where significant differences were found at all time points except 6 h. These data show sympathetic neurons respond to both low and high dose anti-NGF with a similar increase in neurite length as that seen in axotomized ganglia.

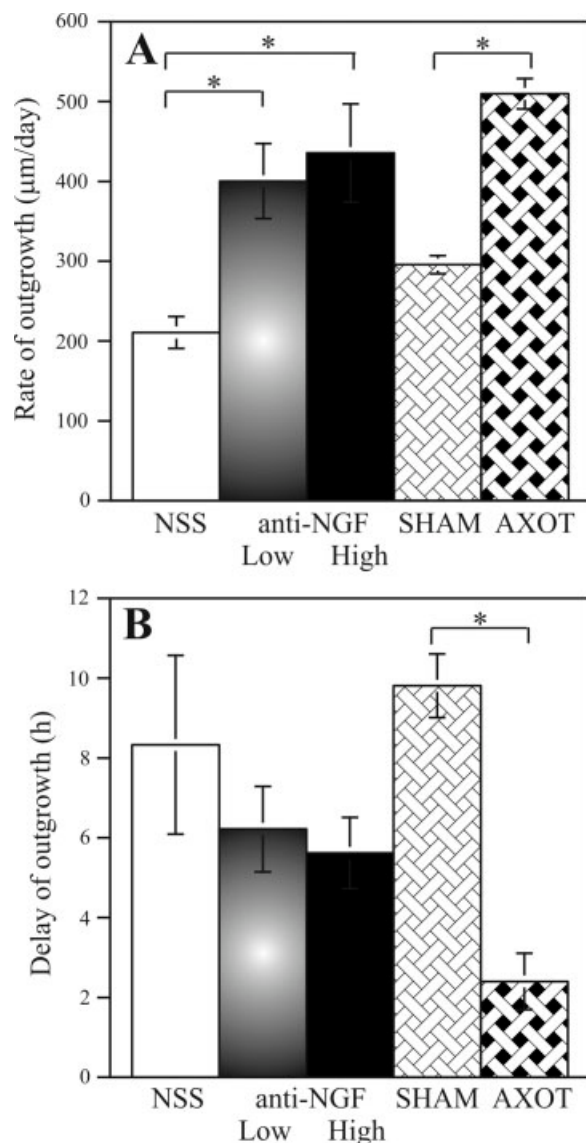


Figure 3 Effect of anti-NGF or axotomy on the rate and delay of neurite outgrowth. The rate of outgrowth and delay in the onset of neurite outgrowth were estimated as described in Materials and Methods. Using one-way ANOVA, the mean rate was shown to increase approximately twofold following anti-NGF or axotomy (A). The mean delay decreased following axotomy, but was not significantly changed following anti-NGF treatment (B). Asterisks represent significant differences between the two groups indicated by brackets ($p < 0.05$).

axotomized ganglia ($510 \pm 19 \mu\text{m/day}$) compared to sham-operated ganglia [$296 \pm 11 \mu\text{m/day}$; Fig. 3(A)]. When the rate of outgrowth from ganglia isolated from mice injected with either dose of anti-NGF was compared to that from axotomized ganglia, no significant differences were found. As shown previously (Shoemaker et al., 2005a), the initial delay of out-

growth was significantly decreased from $9.8 \pm 0.8 \text{ h}$ in sham-operated ganglia compared to $2.4 \pm 0.7 \text{ h}$ in ganglia axotomized 1 week prior to explantation [Fig. 3(B)]. In contrast, ganglia isolated from mice injected with either dose of anti-NGF showed no significant change in the initial delay of outgrowth compared to that of mice injected with NSS [Fig. 3(B)].

Prior treatment with anti-NGF also increased neurite outgrowth of SCG neurons in dissociated cultures. Following 24 h in culture, the length of the longest neurite of the βIII tubulin-IR cells was measured. Neurite length from neurons obtained from anti-NGF-treated mice was significantly longer than that from neurons from NSS-treated animals [233 ± 8.7 vs. 165 ± 7.3 ; Fig. 4(A)]. When the five longest neurites per SCG were measured, a 1.9-fold difference was found between experimental and control animals [513 ± 50.3 vs. 265 ± 10.6 ; Figs. 4(B) and 5]. The distribution of the length of processes was plotted. The percentage of neurons with neurite length in the range of 100 to 199 μm was significantly greater from control neurons than from neurons obtained from anti-NGF-treated animals, and almost all of the neurons with processes in excess of 300 μm were from the anti-NGF-treated animals [Fig. 4(C)]. There was no difference in the percentage of βIII tubulin-IR cells with neurites between the anti-NGF and NSS groups [43.6 ± 3.1 vs. 47.6 ± 2.1 ; Fig. 4(D)].

To examine whether treatment of mice with anti-NGF or axotomy of the SCG affected the levels of NGF in the ganglion, NGF protein concentrations were measured using ELISA. Axotomized ganglia exhibited a significant decrease in NGF levels compared to that of sham-operated mice ($0.2 \pm 0.2 \text{ pg}$ and $7.5 \pm 0.7 \text{ pg}/\mu\text{g}$ protein, respectively). Ganglia isolated from NSS-injected mice contained $4.9 \text{ pg}/\mu\text{g}$ NGF and this level was decreased to undetectable levels in ganglia isolated from anti-NGF-injected mice. Undetectable levels of NGF were observed in ganglia from anti-NGF-injected mice even when the amount of protein loaded for lysates from SCG from anti-NGF-treated animals was six times larger than that of lysates from SCG of NSS-injected mice (3.0 vs. $0.5 \mu\text{g}$, respectively).

Our data indicate that the increased rate of outgrowth induced by anti-NGF is similar to that observed following axotomy. If decreased NGF levels in the SCG were required for the axotomy-induced conditioning lesion effect, it might be possible to inhibit that effect by administration of exogenous NGF. To examine this possibility mice were injected daily for 1 week with $\beta\text{-NGF}$ ($1.0 \mu\text{g/g}$) or with a control protein, cytochrome C ($1.0 \mu\text{g/g}$), starting immediately prior to axotomy and sham operations.

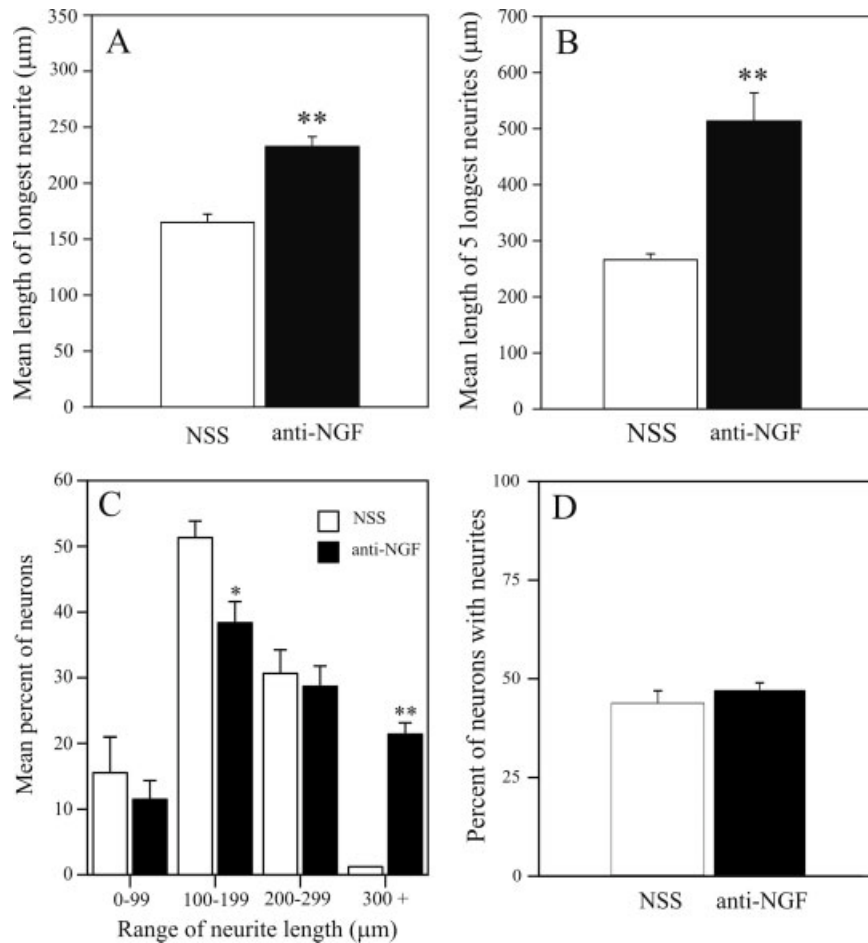


Figure 4 Effect of anti-NGF on sympathetic neurite outgrowth from dissociated SCG neurons after 24 h in culture. (A) The mean length of the longest neurite per neuron was significantly greater in dissociated neurons from anti-NGF-injected mice (black bars) than in neurons from NSS-injected mice (open bars). More than 300 neurons were measured for each treatment. (B) The mean of the five longest neurites per animal from NSS-injected mice compared to that of anti-NGF-injected mice. (C) Cells were subdivided into four ranges of neurite length and the percent of the total number of cells in each range was determined for each treatment group. The mean percent per treatment group was compared in each range. (D) There was no difference in the percentage of neurons with neurites between treatment groups. * $p < 0.01$, ** $p < 0.001$.

As found in ganglia from uninjected mice, ganglia from mice injected with cytochrome C showed a significant increase in neurite length after axotomy at all time points measured (Fig. 6). Exogenous NGF had no effect on neurite length in sham-operated ganglia compared to that in ganglia from mice injected with cytochrome C. Axotomized ganglia from mice injected with NGF showed a slight trend for a decrease in neurite outgrowth, but no consistent differences in the mean neurite length were found (Fig. 6).

The concentration of NGF in SCG from NGF- and cytochrome-C-injected mice was measured by ELISA. A significant increase in NGF protein levels was detected in sham-operated SCG of mice treated

with exogenous NGF (13.5 ± 2.0 pg/ μ g protein) compared with those from cytochrome-C-injected mice (8.1 ± 0.6 pg/ μ g protein; Fig. 7). However, no significant difference was found in NGF levels between axotomized ganglia from NGF-injected and control mice (2.9 ± 1.9 and 0.2 ± 0.2 pg/ μ g protein, respectively; Fig. 7).

DISCUSSION

Following axonal injury, marked physiological, morphological, and biochemical changes occur in axotomized peripheral neurons. In considering the types

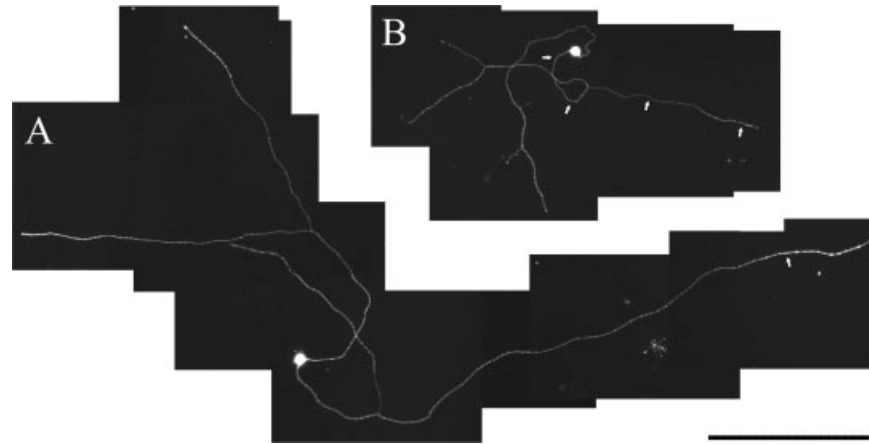


Figure 5 Montages of fluorescence photomicrographs of dissociated SCG neurons immunoreactive for β III tubulin. These examples are representative of the mean length of the five longest neurites per animal [see Fig. 4(B)]. The longest process of a dissociated SCG neuron from an anti-NGF injected mouse [515 μ m; (A)] is approximately twice as long as that of a neuron from a NSS-injected mouse [258 μ m; (B)]. Arrows indicate the longest process of the neuron. Scale bar = 100 μ m.

of molecular signals that might trigger such changes, Cragg (1970) suggested that one such signal might be reduced availability of a target-derived trophic factor to the axotomized cell body. Initial support for this hypothesis came from the findings of Pilar and Landmesser (1972) and Purves (1976), that the synaptic depression that occurs in autonomic ganglia after postganglionic axotomy can be elicited in intact animals by local blockade of axonal transport. Nja and Purves (1978) subsequently showed that synaptic depression (and the ultrastructural changes that accompany it) is also produced in intact preparations after systemic administration of an antiserum against NGF, providing evidence for the identity of one such trophic factor. Similarly, with respect to the characteristic axotomy-induced changes in gene expression that occur in sympathetic and some sensory neurons, certain of these changes can be produced either with inhibitors of axonal transport (Keen et al., 1989; Knyihar-Csillik et al., 1991a; Kashiba et al., 1992; Zigmond et al., 1996) or with anti-NGF (Knyihar-Csillik et al., 1991b; Gold et al., 1993; Shadiack et al., 1998, 2001; Hyatt Sachs et al., 2005).

The first suggestion that a target-derived inhibitory molecule might trigger the conditioning lesion effect after axotomy came from the observation of enhanced neurite outgrowth in cultured sensory neurons taken from animals whose sciatic nerves had been treated with inhibitors of axonal transport (White et al., 1996; Smith and Skene, 1997). In apparent contrast, however, Kanje et al. (1991) found that application of the inhibitor vinblastine produced no conditioning lesion-like effect *in vivo* and in fact blocked the conditioning lesion effect produced by

sciatic nerve crush. In the present study, we demonstrate that treatment with NGF antiserum *in vivo* produces a conditioning lesion-like effect on sympathetic

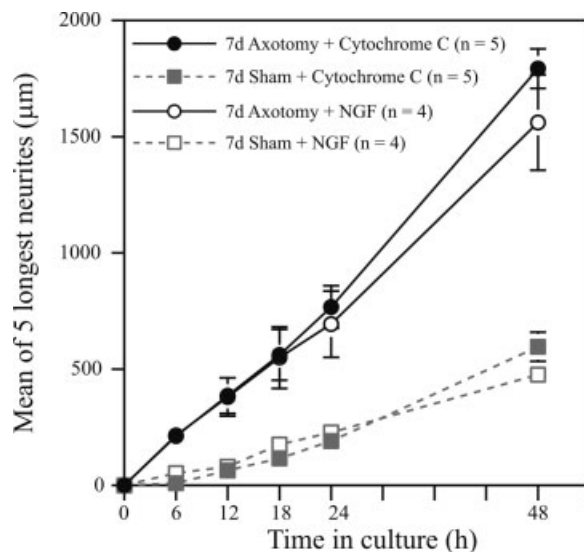


Figure 6 Effect of exogenous NGF on the conditioning lesion effect induced by axotomy. The SCG was unilaterally axotomized and 1 μ g/g NGF or cytochrome C (CytoC) was injected daily. After 1 week, axotomized and sham-operated ganglia were explanted in Matrigel. Neurite length from axotomized ganglia from both CytoC- and NGF-injected mice was significantly increased compared to that from the respective sham-operated ganglia. Ganglia from mice injected with NGF exhibited no significant difference in the conditioning lesion effect or in the neurite length of sham-operated ganglia compared to that of animals injected with CytoC. n = the number of explants included in each experimental group.

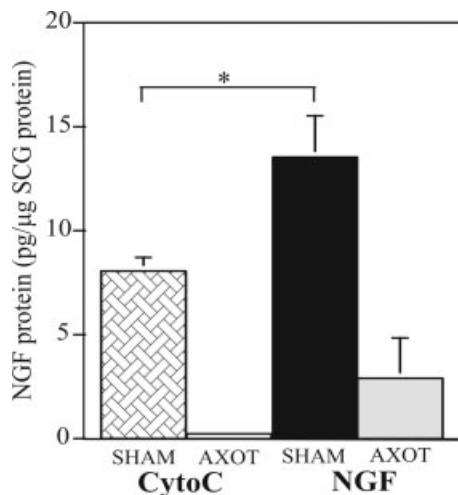


Figure 7 NGF protein levels in the sham-operated and axotomized SCG following exogenous NGF. The concentration of NGF in mouse SCG lysate was measured using ELISA. The mean concentration of NGF significantly increased in sham-operated ganglia of mice injected for 1 week with exogenous NGF compared to that of cytochrome C (CytoC)-injected mice. No significant difference in the concentration of NGF protein was observed between axotomized ganglia of mice injected with NGF or CytoC. Comparisons between groups were analyzed by performing a one-way analysis of variance (ANOVA) followed by a multiple comparisons test using the Student-Newman-Keuls method. Asterisk represents a significant difference between the two groups indicated by brackets ($p < 0.05$).

neurite outgrowth in both explant and dissociated cell culture. While considerable evidence exists that NGF promotes axonal regeneration (discussed below), our data suggest that the reduction in NGF availability after axotomy functions as a signal to trigger regeneration.

Role of NGF and Axotomy in Sympathetic Neuron Survival

The role of NGF in the functioning of sympathetic and some sensory neurons is well known to vary depending on the stage of maturity of the organism. Developing sympathetic neurons require NGF for survival, and extensive neuronal cell death occurs following treatment of neonatal animals with an anti-serum against NGF (Levi-Montalcini and Booker, 1960), following culturing of neonatal neurons in the absence of NGF (Levi-Montalcini and Angeletti, 1963; Chun and Patterson, 1977), or in mice in which the genes for NGF or its receptor *trkA* have been knocked out (Crowley et al., 1994; Smeyne et al., 1994). While some cell loss also occurs in adult sympathetic neurons after prolonged NGF de-

privation *in vivo* and while there is marked atrophy of the cell soma under these conditions, many neurons survive in the adult (Angeletti et al., 1971; Bjerre et al., 1975; Gorin and Johnson, 1980; Ruit et al., 1990). Cultured adult sympathetic neurons are also much less dependent on NGF than are neonatal sympathetic neurons (Johnson, 1983; Orike et al., 2001b; Shoemaker et al., 2005a; Fig. 4).

In vivo, sympathetic neurons obtain most of their NGF from their target tissues as evidenced by the existence of a retrograde transport system for the neurotrophin (Hendry et al., 1974; Stockel et al., 1974), by the accumulation of the factor distal to an axonal ligature (Korsching and Thoenen, 1983; Palmatier et al., 1984), and by the substantial decrease in ganglionic NGF levels after surgical and chemical axotomy (Korsching and Thoenen, 1985; Zhou et al., 1994; Nagata et al., 1987; present study). Decreased NGF availability is thought to be the main cause of the rapid neuronal cell death that occurs after axotomy of neonatal SCG (Hendry, 1975; Snider et al., 1992). Significant cell loss also occurs in adult ganglia after a prolonged period of time following axotomy (Purves, 1975) or following transection extremely close to their cell bodies (Hou et al., 1998); however, many neurons survive and successfully reinnervate their target tissues (e.g., Langley, 1895; Purves and Thompson, 1979).

While target tissues are the main source of NGF for sympathetic neurons, they are not necessarily the only sources. In fact, after axotomy, NGF expression increases in Schwann cells in the distal stump of the sciatic nerve (Bandtlow et al., 1987; Heumann et al., 1987; Raivich et al., 1991). It was originally proposed by Johnson and colleagues that the up-regulations of NGF expression and of the low affinity neurotrophin receptor by Schwann cells after axotomy were part of a mechanism that led to the neurotrophin being "transferred to the NGF receptors of regenerating axons" and to the promotion of regeneration (Taniuchi et al., 1986, p 4097). However, to our knowledge, there is no evidence NGF produced by denervated Schwann cells promotes peripheral nerve regeneration. In fact, it is of interest that axotomized sensory neurons decrease their high affinity binding sites for and their retrograde transport of NGF (Verge et al., 1989; Raivich et al., 1991). Whatever sources of NGF might be available to the regenerating sympathetic neuron, our data indicate only low levels remain in the SCG after axotomy or after treatment with anti-NGF. It is also noteworthy that sympathetic neurons, like sensory neurons, decrease their expression of mRNA for both *trkA* and the low affinity neurotrophin receptor *p75* after axotomy

(Verge et al., 1992; Boeshore et al., 2004), suggesting a decrease in the neurons' ability to utilize NGF even if it were available.

Effects of NGF on Sympathetic Neurite Outgrowth and Regeneration

The effect of NGF on neurite outgrowth in adult sympathetic and sensory neurons has been controversial. It has been suggested that exogenous NGF promotes regeneration of axotomized sympathetic neurons (Bjerre et al., 1973; Aloe et al., 1985); however, these studies have depended on visualizing regenerating neurons by catecholamine fluorescence. The interpretation of such experiments is complicated by the facts that axotomy decreases and NGF increases catecholamine biosynthesis (e.g., Paravicini et al., 1975; Federoff et al., 1992). Administration of NGF in nerve conduits has been shown to increase regeneration of sensory neurons from the transected sciatic nerve (Rich et al., 1989; Lee et al., 2003) and dorsal roots (Houle and Johnson, 1989), although it has been proposed that this may represent an indirect effect on regeneration via an effect on non-neuronal cells (Raivich et al., 1991; Houle, 1992; Mohiuddin et al., 1999). In contrast, other studies found that exogenous NGF has no effect on regeneration of sensory neurons (Saunders, 1972; Rich et al., 1984) or in fact delayed their regeneration (Gold, 1997; Young et al., 2001; Hirata et al., 2002). In related studies, it has been shown that exogenous NGF provided to axotomized sensory neurons decreased the induction of growth-associated protein-43 (Mohiuddin et al., 1999; Hirata et al., 2002), a structural protein whose expression is correlated with axon regeneration (Skene and Willard, 1981) and is required for neurite outgrowth (Jap Tjoen San et al., 1992).

If endogenous NGF played an important role in regeneration, one would expect that anti-NGF would inhibit peripheral nerve regeneration. However, studies on both sympathetic and sensory neurons indicate that regeneration is unaffected by anti-NGF under conditions in which collateral sprouting is inhibited (Diamond et al., 1987, 1992; Gloster and Diamond, 1992, 1995; Doubleday and Robinson, 1995). Our data extend these findings by demonstrating that anti-NGF actually triggers a propensity for regeneration in otherwise uninjured sympathetic neurons and suggest that the reduction in NGF availability after axotomy is sufficient to trigger the conditioning lesion effect.

In an attempt to determine whether a reduction in NGF levels was necessary for the elicitation of a conditioning lesion effect, NGF was administered to

animals whose SCG was axotomized unilaterally. While the growth factor treatment increased NGF levels substantially in the contralateral control SCG, there was no significant increase in NGF levels in the axotomized SCG. This finding raises the possibility that most of the uptake of NGF by the intact SCG after systemic administration occurs via their sympathetic nerve terminals, which degenerate after axotomy. Uptake of NGF directly by the sympathetic cell bodies in both the intact and axotomized ganglion may be blocked by a blood-ganglion barrier (Depace, 1982; Chau and Lu, 1995), although existence of such a barrier in the SCG has been questioned (Jacobs, 1977; Arvidson, 1979). An additional factor limiting uptake by the axotomized ganglion may be the decreased expression of NGF receptors, both *trkA* and *p75*, that occurs after axotomy (Boeshore et al., 2004). Whether successfully increasing the concentration of NGF in the axotomized ganglion would prevent the conditioning lesion effect or whether other axotomy-induced changes (e.g., the increased expression of cytokines) would be sufficient to maintain the regenerative response remains to be determined.

As with the *in vivo* data, the extent of the dependence on NGF of neurite outgrowth from adult sympathetic neurons in culture has varied in different studies. Campenot (1981) found that after neonatal sympathetic neurons had been maintained in culture for 20 days in the presence of NGF, they could regenerate neurites following neuritotomy in the absence of NGF, although the density of neurites formed was higher in the presence of NGF. Orike et al. (2001b) found that when adult rat sympathetic neurons were placed in culture only 8% of them produced processes in the absence of NGF, while 54% did in the presence of NGF. In apparent contrast, we have found that greater than 40% of adult mouse sympathetic neurons produce processes in the absence of exogenous NGF (Fig. 4; Shoemaker et al., 2005a). In explants of adult rat SCG, Niwa et al. (2002) reported very little fiber outgrowth into collagen and marked stimulation following addition of NGF; however, in our study, substantial neurite outgrowth occurs in Matrigel in the absence of added NGF (Figs. 1 and 2; Shoemaker et al., 2005a).

One important distinction in studying neurite outgrowth is that made between neurite elongation and neurite branching. Smith and Skene (1997) classified process outgrowth from dissociated sensory neurons as either "branching" or "elongating". The authors found that a conditioning lesion prior to dissociation of sensory ganglia increased the "elongating" population of neurons. These results are similar to those in our study in which we showed an increase in the rate

of neurite elongation following a conditioning lesion. *In vitro*, NGF promotes branching and has no effect on elongation of adult sensory neurons (Yasuda et al., 1990; Gavazzi et al., 1999). Recently, Glebova and Ginty (2004) suggested a similar role for NGF in developing sympathetic neurons. Using NGF/Bax null mice, they showed that sympathetic neurons extend axons towards their targets in the absence of NGF; however, axons fail to innervate and form synaptic terminals within the targets. Altogether, these studies support the idea that sprouting, branching, and target innervation require NGF, but that the process of elongation of axons is NGF-independent.

Interpretation of the results observed with anti-NGF treatment relies on the specificity of antibody to NGF and the efficiency of anti-NGF in inhibiting NGF signaling. The specificity of anti-NGF has been assessed *in vitro* by showing the antiserum to NGF used in the present study blocks neurite outgrowth promoting effects of NGF and neurotrophin-3 (NT-3), but not brain derived neurotrophin factor on neonatal sensory neurons (Van der Zee et al., 1995). Thus, we cannot rule out the possibility that part of the effect of the antiserum might be via inhibition of NT-3 action. NT-3 is retrogradely transported by sympathetic neurons (Zhou et al., 1997) and is required for survival of sympathetic neurons during development (Ernfors et al., 1994, 1995; Zhou and Rush, 1995; ElShamy et al., 1996; Tafreshi et al., 1998; Francis et al., 1999). Little is known about the actions of NT-3 on sympathetic axon outgrowth, though the neurotrophin has been found to enhance neurite outgrowth in adult SCG explanted in a collagen gel (Niwa et al., 2002) and has been proposed to function in proximal axon extension in developing sympathetic neurons (Glebova and Ginty, 2005).

The conditioning lesion response observed after cutting the internal and external carotid nerves was comprised of both an increase in the rate of neurite outgrowth and a decrease in the delay prior to outgrowth (Shoemaker et al., 2005a). In contrast, anti-NGF treatment increased the rate of outgrowth but did not change the initial delay. This lack of a change in delay was similar to sympathetic neurons' response to a distal conditioning lesion (i.e., removal of a target tissue; Shoemaker et al., 2005a). While the basis behind this difference in the regulation of delay is unclear, it has been observed in a number of studies that the cell body response to a proximal lesion can differ from that to a distal lesion (e.g., Lieberman, 1971; Doster et al., 1991). Our data suggest some, as of yet undetermined, aspect of the proximal lesion influences neurons to begin to extend neurites earlier than is seen in neurons in ganglia

from sham-operated mice, distally lesioned mice, or anti-NGF-treated mice.

As in neurons from SCG explants, dissociated SCG neurons from mice pretreated with anti-NGF also showed an increase in mean neurite length. The fact that anti-NGF selectively affected the percentage of neurons with the longest neurites (i.e., longer than 300 μm ; Fig. 5) suggests that not all sympathetic neurons were affected equally. Interestingly, we have recently found that another effect of anti-NGF treatment, namely the induction of ATF3 immunoreactivity, also takes place in a subpopulation of SCG neurons following anti-NGF treatment (Hyatt Sachs et al., 2005). It is not known whether the affected neurons project to a particular target and, for some reason, are more sensitive to anti-NGF treatment or whether the concentration of anti-NGF used is not producing a maximal effect.

Possible Mechanisms Underlying the Effects of Reduction of NGF Availability

It is of interest to understand the signaling downstream of NGF that inhibits the regenerative mode of neurons in intact adult neurons, for example whether it involves activation of trkA or p75. It is noteworthy that the activation of p75 by exogenous brain derived neurotrophic factor has been shown to inhibit neurite outgrowth in neonatal sympathetic cultures, whereas inhibition of p75 by the addition of a function-blocking antibody dramatically enhanced the density of NGF-induced neurite outgrowth (Kohn et al., 1999). The inhibitory effects of p75 activation on neurite outgrowth are also supported in *in vivo* studies showing that p75-null mice exhibit enhanced NGF-induced sprouting of sympathetic axons (Walsh et al., 1999). It would be of interest to inhibit p75 in adult sympathetic neurons prior to axotomy and then examine the effect on sympathetic neurite outgrowth. We propose that the interaction of NGF and p75 may inhibit sympathetic neurons from entering a regenerative mode and predict that inhibition of NGF binding to p75 would induce a conditioning lesion-like effect.

An additional major question to be answered is what biochemical changes produced by neurotrophin withdrawal are critical for the increased regenerative response observed in sympathetic neurons. While much is known about the mechanism of NGF action on sympathetic neurons, much less is known about the mechanisms induced by NGF withdrawal. PC12 cells respond to NGF withdrawal by sustained activation of the c-JUN NH2-terminal protein kinase and p38 enzymes and inhibition of extracellular regulated

kinases (ERKs), changes that have been associated with apoptosis (Xia et al., 1995). At the transcriptional level, certain changes in gene expression in developing sympathetic and sensory neurons resulting from NGF deprivation have been associated with neuronal cell death (for review see Freeman et al., 2004). Interestingly, it has been suggested previously that there might be some overlap between gene induction that is associated with neuronal cell death during development and gene induction that is associated with regeneration in the adult (Herdegen et al., 1997). As noted already, treatment with anti-NGF produces certain changes in gene expression in sympathetic neurons that are also seen after axotomy (Shadiack et al., 1998, 2001; Boeshore et al., 2004), although it seems likely that it only produces a subset of the latter changes. At present, the functional significance of most of these changes in axon regeneration is not known; however, one such change that may be of interest is the increased expression of ATF3 that occurs in neurons in the SCG both after axotomy and after administration of anti-NGF (Hyatt Sachs et al., 2005). Overexpression of ATF3 in neonatal SCG explants has been shown to increase survival and neurite outgrowth in NGF-deprived cultures (Nakagomi et al., 2003), raising the possibilities that this transcription factor may play a role in regeneration in adult sympathetic neurons and may be involved in the conditioning lesion effect.

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